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**13. ABSTRACT (Maximum 200 Words)**
In the past year, our work focused on defining the functional impact of specific caspase deficiency as proposed in aim 3. We demonstrated that caspase-3 had feedback action on cytochrome c release in TNF-$\alpha$ treated cells, as it was observed in doxorubicin treated cells. We also found that functional caspase 3 contributed to the up regulation of Fas in the cells treated with chemotherapeutic agents. To study the interactions between caspase-3 and bcl-2, we have established bcl-2 overexpression cell lines with or without caspase-3 expression, and found that high levels of caspase-3 and bcl-2 were not compatible. To study the specific role of caspase-3 and p53 dependent apoptosis, using SiRNA technology, we have established p53 knock out cell lines form MCF-7/pv, MCF-7/caspase 3 and MDA-MB-231 cells. We also studied the role of caspase 10 in chemotherapy responsiveness by examining the sensitivity of control and caspase-10 reconstituted MCF-7 cells to doxorubicin and etoposide. With our collaborators, we studied down regulation of caspase-3 in human breast cancers. The results and the cell lines generated in last year will facilitate the accomplishment of the proposed project.

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**Introduction**

Apoptosis (programmed cell death) is a fundamental process involved in homeostasis and the biochemical responses to different anti-tumor therapies. Aberrant expression of other apoptotic regulators, such as Bax-α and p53, has been associated with carcinogenesis and therapeutic resistance of breast cancer. Based on the fact that caspases are a group of proteases that mediate apoptotic execution, and that caspase 3 is deficient in MCF-7 breast cancer cells, this project was proposed to study the significance of caspase deficiency in breast cancer carcinogenesis and resistance. The specific aims are:

1. To determine the incidence and pattern of caspase deficiencies in breast cancer via screening for specific caspase expression in breast cancer derived cell lines, explant cultures and snap frozen human breast cancer tissue.

2. Correlation of caspase data (obtained in aim 1) with apoptosis induction using immune modulators (TNF-α, anti-Fas, GrB/Ad), chemotherapeutic agents and radiation on breast cancer derived cell lines and explant cultures.

3. Define the biological role of specific caspase deficiencies via reconstitution of deficient caspases and comparative studies to define apoptosis induction in response to immune modulators, chemo- and radiation therapy.

This grant was supposed to be finished by this year. Because of the change of institution in previous year, the progress of the project was somewhat delayed. We have requested no cost extension for another year, which has been approved. Therefore, this report is prepared as a regular annual report.

**Body**

In the fourth year of this project, our main focus was on the Aim3 of the original proposal, which was reconstitution of the deficient caspases and comparative studies to define apoptosis induction in response to immune modulators, chemo and radiation therapy.

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1. Feedback action of caspase 3 on cytochrome c release from the mitochondria of the cells treated TNF-α.

In last year's report, we showed that caspase 3 had feedback action on apical factors, including apical caspases and cytochrome c release in doxorubicin treated cells. We also detected caspase 3 mediated feedback action on apical caspases in the cells treated with TNF-α. To complete this mini-project, we then measured cytochrome c release from the mitochondria of the cells treated with TNF-α. (Figure 1). After three hours treatment of the cells with 10 ng//ml TNF-α plus 2 ug/ml cycloheximide (CHX), cytochrome c released from mitochondria increased in both MCF-7/PV and MCF-7/C3 cells, as compared to untreated cells. The cytosolic cytochrome c levels inTNF-α treated MCF-7/c3 cells, however, was significantly higher in the treated MCF-7/C3 cells than that of the MCF-7/PV cells. The result demonstrated that caspase 3 also had feedback action on cytochrome C release in the cells treated with TNF-α, suggesting that this was a relatively universal phenomenon. We are in the middle of preparing the manuscript on caspase 3 mediated feedback action.

2. Effect of caspase 10 reconstitution on chemosensitivity of MCF-7 cells.

Based on the original proposal, we plan to reconstitute deficient caspases in appropriate breast cancer cell lines and study their sensitivity to therapeutic agents. From the screening assay, we found that, although the levels of individual caspases varied from cell line to cell line, complete deletion of certain caspase was not common among the breast cells we detected. Nevertheless, very low levels of caspase-10 was detected in MCF-7 cells. We then established MCF–7/C10 cells by overexpressing caspase-10 in MCF-7 cells and studied their responsiveness to doxorubicin an etoposide. As shown in Figure 2, reconstitution of caspase 10-A significantly sensitized MCF-7 to etoposide, as compared to the control cells. The sensitization of the cells to doxorubicin, however, was marginal. In context with our previous results, which showed that caspase-3 reconstitution mediated sensitization to doxorubicin was more significant than etoposide, it suggests that there might be some discrepancies among different chemo therapeutic agents. The mechanism of caspase 10 reconstitution mediated sensitization to chemo drugs remain to be studied. Further characterization of caspase-10 reconstituted MCF-7 cells will be followed.

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3. Enhanced up regulation of Fas in doxorubicin treated MCF-7/C3 cells.


It was reported that up regulation of Fas expression was detected in breast cancer cells treated with certain chemo drugs (1). Our preliminary results indicated that caspase 3 had feedback action on apical factors. We then asked whether up regulation of Fas was involved in caspase 3 mediated sensitization of MCF-7 cells to chemo therapy drugs. Using our MCF-7/PV and MCF-7/C3 cell line model, we detected the protein levels of Fas in both MCF-7 PV and MCF-7/C3 cells treated with doxorubicin. Probing with a monoclonal antibody specifically against Fas, we detected a protein band that was significantly increased in MCF-7/C3 cells treated with 2-10 µM doxorubicin for 24 hours (Figure 3). Although the result is still preliminary, significant up regulation of Fas in caspase 3 mediated apoptosis in response to chemo therapeutic drugs is very interesting. We will verify this result and perform related mechanism studies.

4. Interaction of caspase 3 and BCL-2 in breast cancer cells.


It has been established that BCL-2 is the pivotal regulator of apoptosis by regulating mitochondria membrane stability, which is upstream of caspase 3. However, it was also reported that active caspase 3 could cleave anti-apoptotic BCL-2 and the cleaved product become pro-apoptotic (2). It appears that the interactions between caspase 3 and BCL-2 is closely related to chemo sensitivity. Since BCL-2 overexpression is frequently detected in breast cancers, we hypothesize that if BCL-2 overexpression is accompanied by caspase deficiency in a breast cancer, it will result in more resistance to chemo-therapies. To test our hypothesis, we overexpressed BCL-2 in caspase 3 deficient MCF-7/PV and caspase 3 reconstituted MCF-7/C3 cells. With great effort, we have established four sets of new sublines by cell cloning. The sublines include MCF-7/PV/control, MCF-7/PV/BCL-2, MCF-7/C3/control and MCF-7/C3/BCL-2. We first detected caspase 3 and BCL-2 levels in a number of selected clones (Figure 4). It was very interesting that high levels of caspase 3 appeared not to be compatible with high levels of BCL-2. Among the selected clones, the cells with

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high levels of BCL-2 overexpression had lower levels of caspase 3, such as clone #18 and #21. The levels of caspase 3 in the cells without BCL-2 overexpression were relatively higher, such as clone #14 and clone #19. With these valuable resources, we will perform in-depth studies on the role of caspase 3/BCL-2-interaction in caspase 3 dependent apoptosis.

5. Establishment of p53 knockout MCF-7/PV and MCF-7/C3 cell lines.

It has been reported that apoptosis induction and execution can be p53 dependent, p53 independent, caspase 3 dependent and caspase-3 independent (3, 4). How much caspase 3 dependent apoptosis contributes to P53 dependent apoptosis remains unclear. We hypothesized that caspase 3 dependent apoptosis play a significant role in p53 dependent apoptosis and therapeutic sensitivities, and breast cancers with both p53 and caspase deficiency would be more resistant to therapeutic agents. To test our hypothesis, we had established p53 deficient MCF-7 cell lines by overexpressing dominant negative p53 in MCF-7/PV and MCF-7/C3 cells, respectively (which was reported in the second year's annual report). Due to the problems of blocking efficiency and possible gain of function associated with p53 mutant, the result from these sublines were not convincing enough.

To overcome these problems in addressing the role of caspase 3 in p53 dependent apoptosis, we established new P53 knockout sublines from MCF-7/PV, MCF-7/C3 and MDA-MB-231 cells using SiRNA technology. As shown in Figure 5, p53 levels were significantly knocked down in the selected clones of each cell line. We have obtained clones of p53 knockout lines from each parental cell line. To test whether p53 knockout cell lines had functional consequences, we treated MCF-7/PV/SRIP53 cells with doxorubicin and detected the protein levels of P53 and its target P21 (figure 6). Our result indicates p53 knockout was efficient both in non-treated cells and the cells treated with DNA damaging agent (doxorubicin). Correspondently, expression of its target gene P21 was also blocked. More importantly, we have tested the chemosensitivity of MCF-7/pv and MCF-7/pv/Rip53 cells and found that the cells with both p53 and caspase-3 deficient (MCF-7pv/Rip53) cells were significantly more resistant to doxorubicin, taxol cisplatin and Etoposide (Fig. 7, Fig 8). We will use these cell lines for further analysis of apoptosis in the cells with p53+/caspase-3+, p53+/caspase-3-, p53-/caspase-3+ and p53-/caspase3.

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6. Down regulation of caspase-3 in breast cancers.

In collaboration with Dr. Kapil Mehta at the M.D. Anderson Cancer Center, we studied caspase-3 expression in breast cancers. As detailed in appendix C, we found that down regulation of caspase-3 was frequently detected in breast cancer cell lines and tissues. Down regulation of other caspases was relatively not significant. The results suggest that down regulation of caspase-3 might contribute to breast cancer development and therapeutic resistance.

## Key research accomplishments

- We demonstrated that caspase-3 has beedback action on cytochrome c release from mitochondria of the cells treated with TNF-a and cycloheximide.

- We have established caspase-10 reconstituted MCF-7 cell line and tested their sensitivity to etoposide and doxorubicin treatments.

- We have examined the Fas expression in doxorubicin treated MCF-/pv and MCF-7/caspase 3 cells.

- We have established Bcl-2 overexpressing cell lines from MCF-7/pv and MCF-7/caspase-3.

- Using SiRNA technology, we have established p53 knockout cell lines from MCF-7/pv, MCF-7/caspase 3 and MDA-MB-231 cells.

- In collaboration with Dr. Mehta at the M.D. Anderson Cancer center, we studied the downregulation of caspase-3 and its significance in breast cancers.

## Reportable Outcomes

Meeting Abstract:

**XiaoHe Yang**, Todd Sladek, Xuesong Liu, Shihe Yang, Brett Ellefson, Janardan khandekar, Christopher Froelich and Ann Thor, Critical role of caspase-3 in MCF-7 breast cancer cells in response to different anti-cancer agents, Era of Hope, The department of Defense Breast Cancer Research Program Meeting, Orlando, Florida, September 2002

Publication:

Devarajan E, Sahin AA, Chen JS, Krishnamurthy RR, Aggarwal N, Brun AM, Sapino A, Zhang F, Sharma D, **Yang XH**, Thor AD, Mehta K, Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. Oncogene;2002, 21:8843-51

**Conclusion**

Results generated in the past year include both conclusive data and ongoing results as listed below.

1. Together with last year'S results, increased cytochrome c release from mitochondria in TNF-α treated MCF-7 cells indicated that caspase-3 mediated feedback action on apical factors, such as apical caspase activation and cytochrome c release, is relatively universal.

2. Sensitization of MCF-7 cells to etoposide ( and doxorubicin to a less degree) by caspas-10 reconstitution suggest that caspase-10 also play a role in chemotherapy induced apoptosis. Caspase-10 deficiency may also contribute to chemo resistance in breast cancers.

3. Our preliminary results suggest that functional caspase-3 may enhance Fas upregulation in doxorubicin treated breast cancer cell. More experiments will be followed to verify this.

4. Although establishment of bcl-2 overexpressing cell lines and p53 knockout cells lines is not a conclusive results, these double transgenic cell lines are valuable resources and will be used for mechanism study in the coming year.

5. In combination with our previous immunohistochemistry data (report of year one), caspase expression results from human breast cancer samples indicated that caspase-3 downregulation/deficiency may contribute to breast carcinogenesis.

**"So what" section**

This project was proposed to study the correlation between caspase deficiency and breast cancer carcinogenesis and therapeutic resistance. The whole grant includes three parts:

A. Detection of caspase expression in breast cancer cell lines and clinical samples.

B. Functional correlation of caspase expression and their sensitivities to different therapeutic

agents.

C. Mechanismatic study the role of specific caspases in response to different stimuli. Basically, the experiments were carried out according to the original design. We have analyzed the expression of 10 caspases in a collection of breast cancer cell lines and performed functional assays on these cell lines (please see pervious reports). The results indicated that caspase deficiency/dow regulation was indeed common among breast cancer cell lines and in breast cancers (referring caspase 3), although down regulation of certain caspases was more common than complete deletion of caspases genes. Functional assays were consistent with general expression levels of caspases. Using MCF-7/pv and MCF-7/c3 cell line model, we have investigated the critical role of caspase-3 in therapeutic resistance and responsiveness.

We would like to point out that caspase related studies is a very fast moving area. For mechanismatic studies proposed in Aim 3 of the original proposal, based on the progress in this field, we had to find novel points pertinent to our aim. This is reflected by slight modification of experimental design when we did it.

In the next year (no cost extension period), we will follow the original proposal to finish the project in the following aspects:

A. Immunohistochemistry detection of caspase expression in breast cancer tissues.

Due to change of institution and IRB protocol application, this part has not been completely done. As we did on caspase-3, we will detect the expression of other caspases in breast cancer tissues.

B. More mechanism study using the cell line models we have established.

Due to sequence homology and functional overlap among different caspases, caspase specific cell line models are very important in detailed analysis of the roles of individual caspase in breast cancer carcinogenesis and therapeutic resistance. Thanks to our MCF-7/pv and MCF-7/c3 cell model, we have performed in depth studies on the role of caspase -3 in breast cancer apoptosis. With the new sub lines we have established, such as MCF-7/caspase-3-/bcl-2, MCF-7/caspase-

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3+/bcl-2 and p53 knock out lines, we will further investigate the critical role of caspase -3 in breast cancer apoptosis. Although it was not reportable outcomes, our cell line models have been requested by many national and international colleagues.

C. Other than the experiments mentioned above, another major task in the nest year is to publish the results generated from this grant. Comprehensive integration of the results in the whole funding period will also be carried out.

## References

1. Villunger A, Egle A, Kos M, Hartmann BL, Geley S, Kofler R, Greil R. Drug-induced apoptosis is associated with enhanced Fas (Apo-1/CD95) ligand expression but occurs independently of Fas (Apo-1/CD95) signaling in human T-acute lymphatic leukemia cells. Cancer Res. 1997 Aug 15;57(16):3331-4.
2. Cheng EH, Kirsch DG, Clem RJ, Ravi R, Kastan MB, Bedi A, Ueno K, Hardwick JM. Conversion of Bcl-2 to a Bax-like death effector by caspases. Science. 1997 Dec 12;278(5345):1966-8.
3. Fulda S, Friesen C, Los M, Scaffidi C, Mier W, Benedict M, Nunez G, Krammer PH, Peter ME, Debatin KM. Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. Cancer Res. 1997 Nov 1;57(21):4956-64.
4. Meng XW, Fraser MJ, Feller JM, Ziegler JB. -3-dependent and caspase-3-independent pathways leading to chromatin DNA fragmentation in HL-60 cells. Apoptosis. 2000 Feb;5(1):61-7.

**APPENDICES:**

**Appendix A:**

Related figures described in body text.
Figures 1 - 8

**Appendix B:** Meeting abstract

Critical role of caspase-3 in MCF-7 breast cancer cells in response to different anti-cancer agents, Era of Hope, The department of Defense Breast Cancer Research Program Meeting

**Appendix C:** Publication

Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. Oncogene; 2002, 21:8843-51

10 ng/ml TNF-α + 2 μg/ml CHX

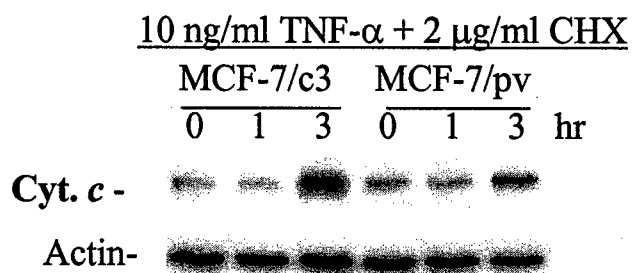| MCF-7/c3 | | | MCF-7/pv | | | |
| 0 | 1 | 3 | 0 | 1 | 3 | hr |

Cyt. *c* -

Actin-

**Figure 1. Feedback action of caspase-3 on cytochrome c release from mitochondria of the cells treated with TNF-α**

MCF-7/pv (caspase deficient) and MCF-7/c (caspase 3 reconstituted ) cells were treated with 10 ng/ml TNF-α plus 2 mg/ml cycloheximide (CHX) for times indicated. Fifty μg of cytosolic extract prepared from the treated cells was separated by SDS-PAGE. Cytochrome c released to cytosol was detected using western blot.
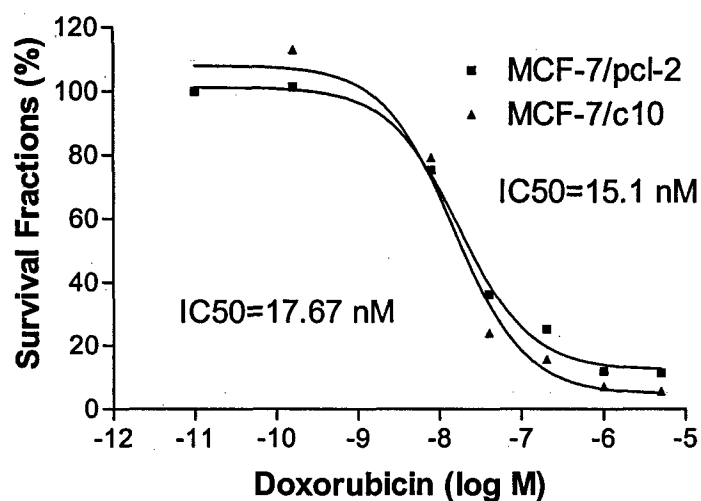
Appendix A
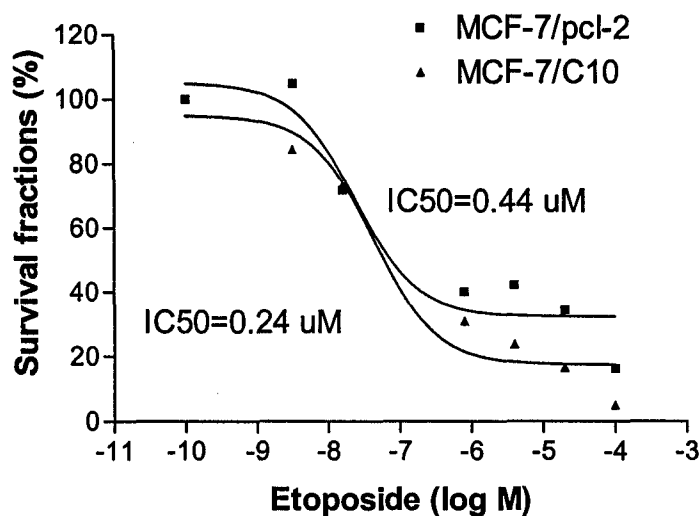


**Fig. 2. Survival fractions of MCF-7/pcl-2 and MCF-7/caspsae-10 cells treated with etoposide and doxorubicin**

Caspase 10 deficient MCF-7/pcl-2 cells and caspase-10 reconstituted MCF-7/caspase-10 cells were treated with etoposide (A) and doxorubicin (B) at the indicated concentration. Survival fractions were determined using MTT assay 6 days post treatment. Each point in the figure is the average survival fractions of three experiments. In each experiment, 8 parallel samples were tested for any concentration treatment. Curve regression and IC50 calculation was generated using Prism software.

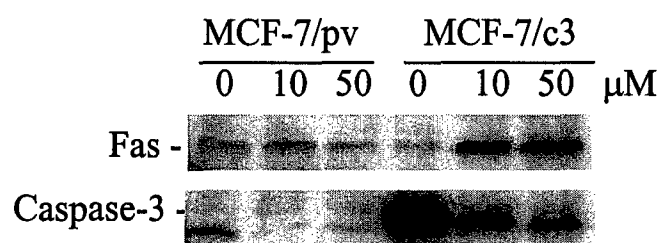|  | MCF-7/pv | | | MCF-7/c3 | | | |
|---|---|---|---|---|---|---|---|
|  | 0 | 10 | 50 | 0 | 10 | 50 | μM |
| Fas - | | | | | | | |
| Caspase-3 - | | | | | | | |

**Figure 3. Upregulation of Fas death receptor in doxorubicin treated MCF-7/c3 cells.**

MCF-7/pv and MCF-7/c3 cells were treated with 10 or 50 μM doxorubicin for 20 hours. The protein levels of Fas was probed using Western blot. Anti-Fas antibody was from Santa Cruz Biotech. Inc. (Fas, N-18)
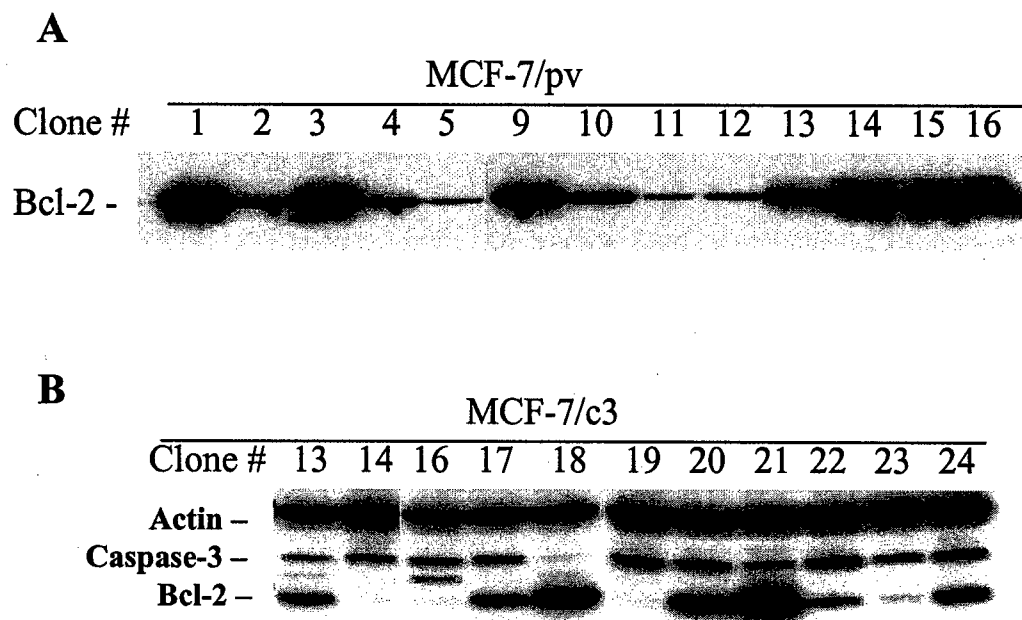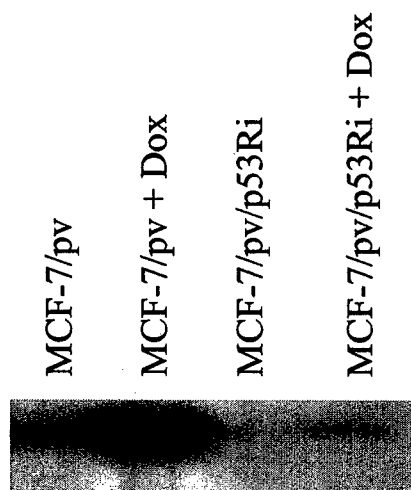
Appendix A

**A**

MCF-7/pv

Clone #   1   2   3     4   5     9   10   11   12   13   14   15   16

Bcl-2 -

**B**

MCF-7/c3

Clone #   13   14   16   17   18   19   20   21   22   23   24

Actin –
Caspase-3 –
Bcl-2 –

**Figure 4. Overexpression of Bcl-2 in MCF-7/pv and MCF-7/c3 cells.**
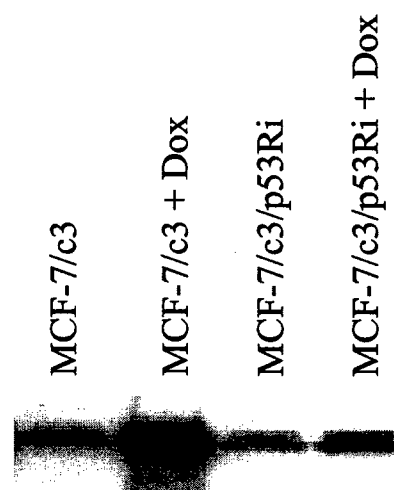
MCF-7/pv (A) and MCF-7/c3 (B) cells were transfected with Bcl-2 encoding
plasmids. G418 resistance cells were cloned. Protein levels of caspase 3, Bcl-2 and
actin were detected using Western blot. After selection and cloning, it appears that
Bcl-2 overexpressing cells had lower levels of caspase-3.

**A**

MCF-7/pv
MCF-7/pv + Dox
MCF-7/pv/p53Ri
MCF-7/pv/p53Ri + Dox

**B**

MCF-7/c3
MCF-7/c3 + Dox
MCF-7/c3/p53Ri
MCF-7/c3/p53Ri + Dox

**C**

MDA-MB-231
MDA-MB-231 + Dox
MDA-MB-231/p53Ri
MDA-MB-231/p53Ri + Dox
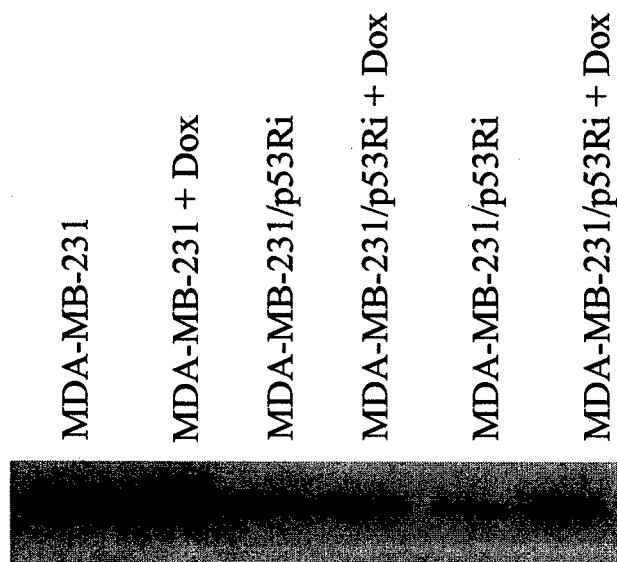MDA-MB-231/p53Ri
MDA-MB-231/p53Ri + Dox

**Figure 5. Establishment of p53 knockout cell lines from MCF-7/pv, MCF-7/c3 and MDA-MB-231 cells.**

MCF-7/pv (A), MCF-7/c3 (B) and MDA-MB-231 (C) cells were transfected with psilencer/Rip53 plasmid and selected with G418. p53 levels of control and Rip53 cells with without DNA damage stimulation (2 µM doxorubicin for 20 hours) were detected using Western blot.
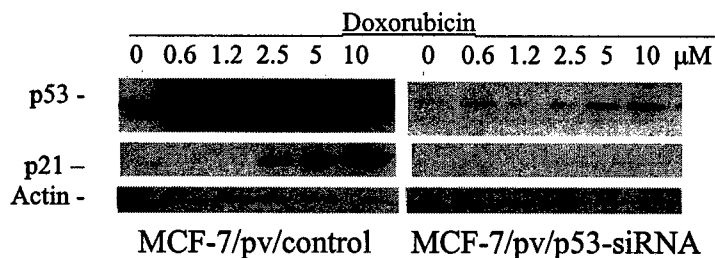
Appendix A



| | Doxorubicin | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 0.6 | 1.2 | 2.5 | 5 | 10 | 0 | 0.6 | 1.2 | 2.5 | 5 | 10 | μM |

p53 -

p21 –
Actin -

MCF-7/pv/control    MCF-7/pv/p53-siRNA

**Figure 6. Efficient knockout of p53 in MCF-7/pv cells using SiRNA technology.**

p53 knock-down in MCF-7 cells using SiRNA. MCF-7/p53-SiRNA cells were prepared
by selecting of MCF-7 cells transfected with p53 targeting SiRNA encoded in pSilencer.
Both cell lines were treated with Doxorubicin at concentration indicated for 20 h. p53 and
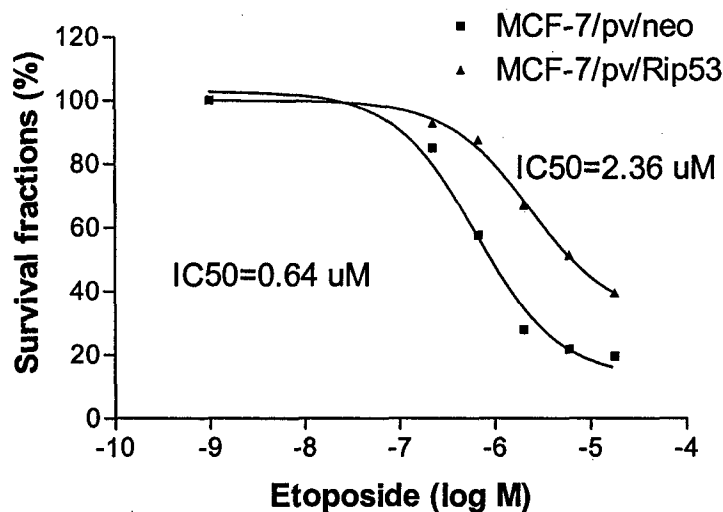p21 in the treated cells were analyzed using Western blot.

**A**
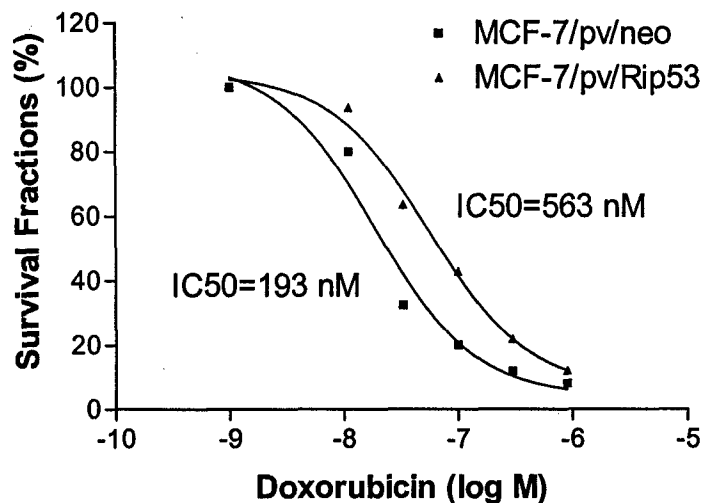


**B**



**Figure 7. Sensitivity of p53 knockout MCF-7/pv cells to doxorubicin and etoposide**

Control (MCF-7/pv/neo) and p53 knockout (MCF-7/pv/Rip53) cells were treated with doxorubicin (A) and Etoposide (B) at the indicated concentrations. Survival fractions were determined using MTT assay 6 days post treatment. Each point in the figure is the average survival fractions of three experiments. In each experiment, 8 parallel samples were tested for any concentration treatment. Curve regression and IC50 calculation was generated using Prism software.

**A**
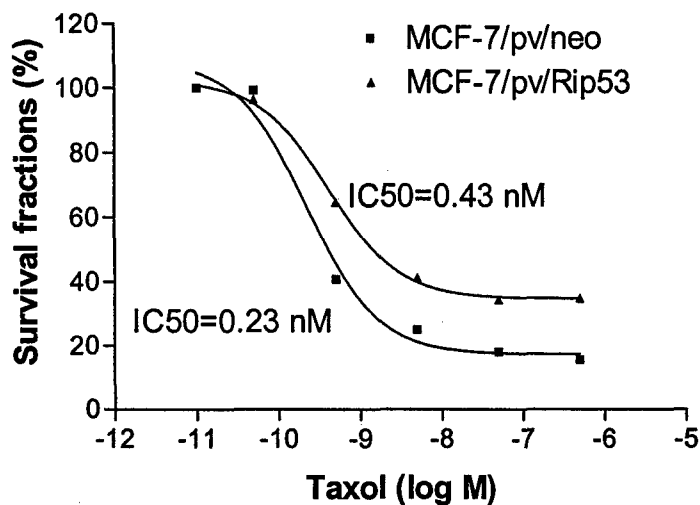


**B**



**Figure 8. Sensitivity of p53 knockout MCF-7/pv cells to taxol and cisplatin**

Control (MCF-7/pv/neo) and p53 knockout (MCF-7/pv/Rip53) cells were treated with taxol (A) and cisplatin (B) at the indicated concentrations. Survival fractions were determined using MTT assay 6 days post treatment. Each point in the figure is the average survival fractions of three experiments. In each experiment, 8 parallel samples were tested for any concentration treatment. Curve regression and IC50 calculation was generated using Prism software.
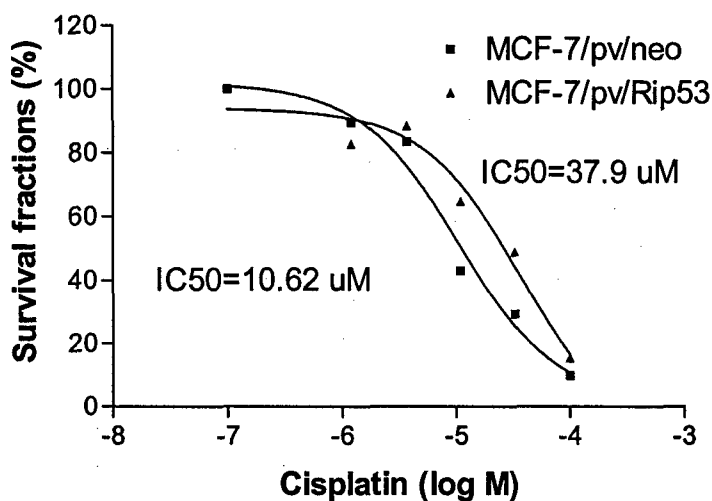
## CRITICAL ROLE OF CASPASE 3 IN MCF-7 BREAST CANCER CELLS IN REPONSE TO DIFFERENT ANTI-CANCER AGENTS

**XiaoHe Yang1,2, Todd Sladek3, Xuesong Liu2, Shihe Yang1,2, Brett Ellefson2, Janardan khandekar2, Christopher Froelich2 and Ann Thor1,2,** 1Department of Pathology, University of Okalahoma Health Science Center; 2ENH Research Institute, Northwestern University; 3Department of Microbiology/Immunology, Chicago Medical School

Apoptosis plays important roles in cellular development and homeostasis. Aberrant apoptosis has been associated with the carcinogenesis and therapeutic resistance of cancer. Although apoptosis can be triggered by different stimuli, the involved signaling pathways ultimately converge to activate a group of proteases, called caspases. MCF-7 breast cancer cells, which are resistant to many apoptotic stimuli, have been found to be caspase 3 deficient. To study the correlation between caspase 3 deficiency and therapeutic resistance, we reconstituted caspase 3 in MCF-7 cells and have now characterized their response to several anti-cancer agents, including chemotherapy, radiation and tumor necrosis factor alpha (TNF-a). Caspase 3 was expressed in MCF-7 cells by transfecting the cells with pBabe/puro retroviral vector encoding caspase 3 cDNA. Flow cytometry and MTT assays showed that caspase 3 expression rendered MCF-7 cells significantly more susceptible to these treatments. Apoptosis medicated by the reconstituted caspase 3 was demonstrated by increased DEVD cleavage activities, activation of effector caspases and cleavage of cellular death substrates. Using this caspases 3 specific cell line model, we found that caspase 7 is downstream of caspase 3 and that caspase 6 could be activated by caspase 3 and other apical caspases. Our results also showed that caspase 3 had feedback signals to apical caspases (8, 9 and 2), as demonstrated by both cell-free and whole cells systems. Enhanced mitochondrial depolarization and cytochrome c release was also detected in caspase 3 expressing MCF-7 cells treated with radiation or doxorubicin. These results demonstrated that caspase 3 plays a central role in apoptosis induced by different anti-cancer agents, suggesting that caspase 3 deficiency might contribute to the therapeutic resistance of MCF-7 breast cancer cells

# Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance

Eswaran Devarajan[1], Aysegul A Sahin[2], Jack S Chen[1], Raghu R Krishnamurthy[3],
Neeraj Aggarwal[3], Anne-Marie Brun[3], Anna Sapino[4], Fan Zhang[1], Dhawal Sharma[1],
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Caspase-3 is a member of the cysteine protease family, which plays a crucial role in apoptotic pathways by cleaving a variety of key cellular proteins. Caspase-3 can be activated by diverse death-inducing signals, including the chemotherapeutic agents. The purpose of this study was to determine the levels of caspase-3 expression in breast tumor samples and to determine whether alterations in its expression can affect their ability to undergo apoptosis. Primary breast tumor and normal breast parenchyma samples were obtained from patients undergoing breast surgery and the expression of caspases-3 was studied. Similarly, normal mammary epithelial cells and several established mammary cancer cell lines were studied for caspases-3 expression by reverse transcriptase-polymerase chain reaction, Northern blot analysis, and Western blot analysis. Approximately 75% of the tumor as well as morphologically normal peritumoral tissue samples lacked the caspase-3 transcript and caspase-3 protein expression. In addition, the caspases-3 mRNA levels in commercially available total RNA samples from breast, ovarian, and cervical tumors were either undetectable (breast and cervical) or substantially decreased (ovarian). Despite the complete loss of caspase-3, the expression levels of other caspases, such as caspase-8 and caspase-9, were normal in all of the tumor samples studied. The sensitivity of caspase-3-deficient breast cancer (MCF-7) cells to undergo apoptosis in response to doxorubicin and other apoptotic stimuli could be augmented by reconstituting caspase-3 expression. These results suggest that the loss of caspases-3 expression may represent an important cell survival mechanism in breast cancer patients.
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## Introduction

Apoptosis is a genetically regulated form of cell death that plays an important role in eliminating infected, damaged, and other unwanted cells from the body (Kerr et al., 1972). With the realization that defects in apoptosis can contribute to diseases like cancer, interest in the control of apoptosis has grown exponentially among cancer researchers. Apoptosis can be triggered by various extracellular and intracellular stimuli that result in coordinated activation of a family of cysteine proteases called caspases.

About 14 caspases have been described so far in mammalian systems (Evan and Littlewood, 1998; Johnstone et al., 2002; Eckhart et al., 2000; Ahmad et al., 1998). On the basis of their role in apoptosis, caspases can be categorized into two major subgroups. The upstream or initiator caspases (e.g., caspase-8, -9 and -10) are activated by apoptotic signals, resulting in the activation of the downstream or executioner caspases (Nicholson, 1999). Following their activation, the executioner caspases, which include caspase-3, -6 and -7, catalyze the specific cleavage of many key cellular proteins, such as poly(ADP-ribose) polymerase, inhibitor of caspase-activated DNase, gelsolin, 4-GDI, $\alpha$- and $\beta$-fodrin, and epidermal growth factor receptor (Thorneberry and Lazebnik, 1998; Cryns and Yuan, 1998). The cleavage of these proteins results in membrane blebbing, chromatin condensation, and DNA fragmentation, the hallmark changes associated with apoptosis. A mitochondrial flavoprotein, apoptosis-inducing factor (AIF), can also induce morphological apoptosis in a caspase-independent manner in response to certain apoptotic stimuli (Cande et al., 2002). Nevertheless, caspase-3 (also known as CPP32, Yama, and apopain) is considered to be the central protein in the execution of apoptosis (Enari et al., 1996) and to play a pivotal role in the development of the central nervous system. For example, caspase-3 knockout mice exhibited decreased apoptosis in the brain and died prematurely (Kuida et al., 1996).

Genetic or epigenetic alterations that disrupt the ability of cells to undergo apoptosis can lead to the

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development of cancer. For example, *p53* is frequently mutated in aggressive and in chemoresistant tumors. The expression of *Apaf-1*, a cell-death effector that acts with cytochrome-*c* and caspase-9 to mediate p53-dependent apoptosis is lost in metastatic melanomas (Soengas *et al.*, 2001). Similarly, the gene for the effector caspase-8 is frequently inactivated in childhood neuroblastomas (Teitz *et al.*, 2000). Moreover, resistance to apoptotic stimuli has been reported frequently in MCF-7 human breast carcinoma cells that lack expression of caspase-3 as a result of a 47 bp deletion in exon 3 of the *CASP3* gene (Janicke *et al.*, 1998; Yang *et al.*, 2001).

Considering the central role caspase-3 plays in executing apoptosis and the observation that several established breast cancer cell lines exhibit altered caspases-3 expression, we examined the expression of caspase-3 in freshly isolated normal and malignant breast tissue samples. Our results revealed some interesting findings; specifically, approximately 75% of the breast tumor samples lacked the caspase-3 transcript and expression of caspase-3 protein, while the remaining samples showed substantial decreases in caspase-3 expression. More interestingly, a similar loss in caspase-3 expression was evident in morphologically normal peritumoral tissue samples obtained from breast cancer patients. These results suggest that loss of caspase 3 expression may represent an important mechanism of cell survival and chemoresistance by breast cancer cells.

## Results

### Caspase-3 expression in breast cancer cell lines

As our first step in determining the levels of caspase-3 in breast cancer cells, we performed Western blot analysis on a panel of human mammary cancer cell lines (Figure 1). Most of the cell lines that we studied contained abundant levels of caspase-3 protein. The three normal human breast epithelial cell lines (MCF10A and the two finite-life-span epithelial cell lines obtained from Clonetics, San Diego, CA, USA) showed considerable levels of caspase-3 protein expression. However, as previously reported (Janicke *et al.*, 1998), MCF-7 cells completely lacked caspase-3 protein expression (Figure 1). Interestingly, two other breast cancer cell lines, BT-20T and ZR-75T, also showed a complete lack of caspase-3 protein expression (Figure 1). Using caspase-3-specific primers, RT–PCR analysis of these two cell lines demonstrated a truncated (approximately 750 bp) transcript that upon sequencing revealed a 125 bp deletion from position 54 in Pro18 to position 178 in Gly60 (unpublished observations). As previously observed (Janicke *et al.*, 1998), the shift in the open-reading frame resulting from this deletion may account for the loss of procaspase-3 protein in BT-20T and ZR-75T cells. Western blot analysis also revealed that the levels of caspases-3 protein expression in some other breast cancer cell lines (Hs854T, HCC1428, BT549, and BT483) were on
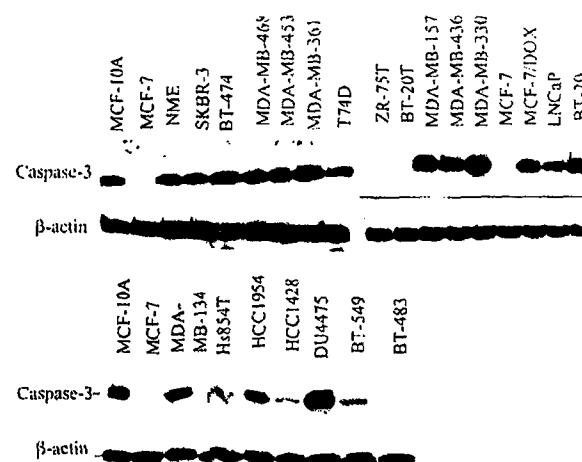


**Figure 1** Levels of caspase-3 protein expression in various breast cancer cell lines were detected using Western blot analysis. Sixty micrograms of sample protein was applied in each lane and fractionated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and probed with an anti-caspase-3 antibody as described in Materials and methods. The membranes were stripped and reprobed with $\beta$-actin antibody to ensure even loading

average five to seven times lower than those in the NME cells (Figure 1).

### Expression of caspase-3 in human breast cancer

In view of the various caspase-3 protein expression levels and transcript sizes in different breast cancer cell lines, we next determined whether similar variations could occur in human breast cancer cells. Breast tissue specimens were collected from a total of 46 patients, including 31 with adenocarcinoma (four of whom had received preoperative chemotherapy), ten with a history of breast cancer, three with benign breast disease, and two undergoing breast reduction surgery. Clinical and pathologic data, including TNM stage, hormone receptor (estrogen receptor (ER) and progesterone receptor (PR)) status, and Her-2 expression levels from the 31 patients with adenocarcinoma are shown in Table 1. Total RNA was extracted from benign and malignant tissue samples, and RT–PCR using caspase-3-specific primers was performed. The results shown in Figure 2a, and Table 1 revealed that about 75% of the breast tumor samples lacked caspase-3 transcript; the remaining 25% had detectable expression levels of caspase-3 mRNA, albeit the levels were significantly lower than those observed in the NME cells.

Morphologically normal breast parenchyma samples obtained from the vicinity of the tumor tissue showed similar lack of or reduction in caspase-3 expression (Figure 2b). Out of the 12 such samples studied (from patients #1–12; Table 1), only one demonstrated detectable caspase-3 expression by RT–PCR analysis. On the other hand, a substantial number (seven out of 10) of normal tissue samples obtained from patients who had a history of breast cancer and had undergone

Table 1   Clinical features of breast cancer patients studied for caspase-3 expression

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| Patient # no. | Patient age, years | TNM stage | Hormone receptor status | | ki-67 | Expression of Her-2 | Casp-3 |
|---|---|---|---|---|---|---|---|
| | | | ER | PR | | | |
| *Breast carcinoma* | | | | | | | |
| 1 | 59 | T1N0M0 | – | – | High | + | – |
| 2[e] | 46 | T4N0M0 | – | – | High | – | – |
| 3 | 68 | T2N1M0 | – | – | High | – | – |
| 4 | 46 | T1N0M0 | + | + | Low | + | – |
| 5 | 49 | T1N0M0 | + | – | Moderate | ND | – |
| 6 | 72 | T2 | + | + | N/A | – | – |
| 7[e] | 56 | T4N0M0 | – | – | Moderate | – | – |
| 8 | 52 | T3N1M0 | – | – | Moderate | – | – |
| 9 | 65 | T0N0M0 | N/A | N/A | N/A | N/A | – |
| 10 | 45 | T2N1M0 | – | – | High | – | – |
| 11 | 64 | T2N0M0 | + | + | Low | – | + |
| 12 | 50 | T3N1M0 | + | – | High | – | – |
| 13 | 52 | T2N1M0 | – | – | High | – | – |
| 14 | 74 | T1N0M0 | + | + | Low | – | + |
| 15 | 59 | T0N0M0 | N/A | N/A | N/A | N/A | + |
| 16 | 40 | T2N2M0 | + | + | Low | – | – |
| 17[e] | 56 | T4N1M1 | – | – | High | – | + |
| 18 | 52 | T1N0M0 | + | + | Low | – | – |
| 19 | 68 | T1N0M0 | + | + | Low | – | – |
| 20[e] | 48 | T2N1M0 | – | – | High | + | – |
| 21 | 56 | T2N0M0 | – | – | High | – | – |
| 22 | 53 | T2N1M0 | – | – | High | – | – |
| 23 | 54 | T2N0M0 | + | + | Low | – | + |
| 24 | 87 | T2N0M0 | – | – | High | – | – |
| 25 | 51 | T2N0M0 | – | – | Moderate | – | – |
| 26* | 60 | T2N0M0 | + | + | High | – | |
| 27 | 59 | T1N0M0 | + | + | Low | – | – |
| 28 | 59 | T1N1M0 | + | + | Low | – | + |
| 29 | 56 | T1N0M0 | + | + | Moderate | – | – |
| 30 | 58 | T4N2M0 | + | – | High | – | + |
| 31 | 38 | T1N0M0 | + | + | Low | – | + |
| *Morphologically normal breast parenchyma (from patients with breast cancer)[a]* | | | | | | | |
| 1 | 59 | | | | | | – |
| 2 | 46 | | | | | | – |
| 3 | 68 | | | | | | – |
| 4 | 46 | | | | | | – |
| 5 | 49 | | | | | | – |
| 6 | 72 | | | | | | – |
| 7 | 56 | | | | | | – |
| 8 | 52 | | | | | | – |
| 9 | 65 | | | | | | – |
| 10 | 45 | | | | | | ! |
| 11 | 64 | | | | | | – |
| 12 | 50 | | | | | | – |
| *(from patients with cancer history)[b]* | | | | | | | |
| 32 | 81 | | | | | | .. |
| 33 | 64 | | | | | | – |
| 34 | 43 | | | | | | + |
| 35 | 45 | | | | | | + |
| 36 | 66 | | | | | | + |
| 37 | 57 | | | | | | + |
| 38 | 56 | | | | | | + |
| 39 | 55 | | | | | | + |
| 40 | 53 | | | | | | + |
| 41 | 43 | | | | | | – |
| *(from patients with non-neoplastic disease)[c]* | | | | | | | |
| 42 | 55 | Hyperplasia | | | | | + |
| 43 | 66 | Hyperplasia | | | | | + |
| 44 | 25 | Fibrocystic | | | | | + |
| 45 | 51 | Breast reduction | | | | | + |
| 46 | 36 | Breast reduction | | | | | + |
| 4* | 46 | Hyperplasia | | | | | + |

[a]Morphologically normal breast parenchyma samples were obtained from the patients #1–12 and tested for caspase-3 expression. [b]Morphologically normal breast parenchyma samples were obtained from patients who had undergone tumor resection surgery in the past. [c]Normal breast tissue samples were obtained from individuals with no history of neoplastic disease. [d]Patient had received chemotherapy prior to the surgery. [e]Tissue sample from the normal side of the breast of patient #4. TNM = tumor, node, metastasis; ND = not done; N/A = not applicable
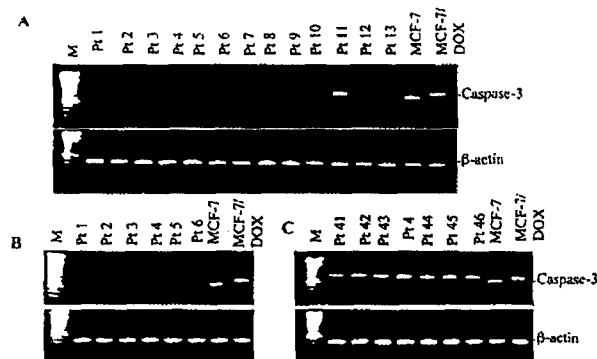
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**Figure 2** RT–PCR analysis of messenger RNA (mRNA) using primers specific for caspase-3 in breast tumor (a), adjacent normal (b), and normal mammary (c) tissue samples. Drug-sensitive (MCF-7) and drug-resistant (MCF-7/DOX) MCF-7 cells were used as positive controls for amplification of the truncated and full-length caspase-3, respectively. Amplification of β-actin was serially analysed for all of the samples to normalize for mRNA integrity and equivalent loading. M represents the 0.1 kb DNA ladder and Pt denotes patient



**Figure 3** Caspase-3 gene expression in human mammary carcinoma (a) and normal tissue samples (b) as determined by Northern blot analysis (a and b) or *in situ* RT–PCR (c). Data for wild-type MCF-7 and drug-resistant MCF-7/DOX breast cancer cell lines, six representative breast carcinomas, and total RNA isolated from human breast, cervical, ovarian, and uterine tumor samples are shown. (b) shows the caspase-3 mRNA expression in various normal human tissues obtained via probing a 12-lane Multiple tissue Northern blot. The S/N ratios indicate the ratio between caspases-3 band and 18S ribosomal RNA (a) or GAPDH (b) band. Pt denotes patient

surgical removal of their tumors in the past (patients #32–41; Table 1) showed detectable levels of caspases-3 expression. As a control, we used the normal breast tissue samples obtained from the patients undergoing breast reduction surgery (patients #45 and 46; Table 1) or from patients with benign breast disease (patients #42–44 and second breast from patient #4; Table 1). All the five normal breast tissue samples showed strong levels of caspase-3 transcript expression (Table 1; Figure 2c).

The loss of caspase-3 expression in breast cancer cells was further confirmed by Northern blot analysis. The results shown in Figure 3, clearly demonstrated that caspase-3 mRNA expression levels in breast tumors were at least 10–50 times lower than those in normal breast tissue or breast cancer cell lines. Specifically, MCF-7 cells showed truncated caspase-3 mRNA due to a 125 bp deletion in the transcript (Janicke et al., 1998). Nevertheless, MCF-7/DOX cells that were derived via continuous culture of MCF-7 cells in the presence of doxorubicin, exhibited a full-length caspase-3 transcript (Figure 3a) as previously demonstrated (Pirnia et al., 2000; Devarajan et al., 2002). We also tested the commercially available total RNA samples that had been isolated from the human breast, cervical, uterine, and ovarian tumors (Clontech Laboratories). To our surprise, the RNA samples isolated from not only the breast tumor sample but also cervical tumor samples, lacked detectable caspase 3 mRNA levels (Figure 3a). The RNA sample from ovarian tumors contained approximately four times lower caspase 3 mRNA than the uterine tumor sample, which was the only tumor sample that we tested, exhibited substantial levels of the caspase-3 transcript. However, a strong signal for caspase-3 mRNA was detected in various normal tissue samples, including the mammary gland, when the human Multiple Tissue Northern blot was probed under identical conditions
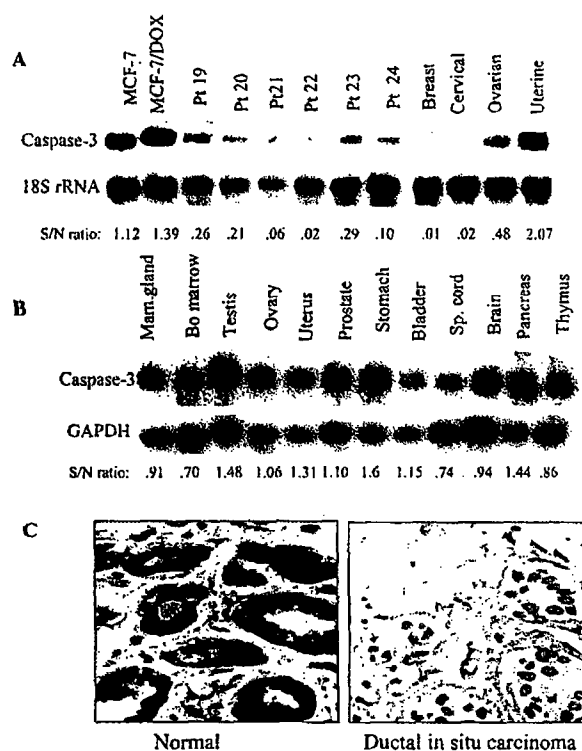
(Figure 3b). The status of caspase-3 transcript expression in breast tissue samples was further determined by *in situ* RT–PCR analysis, using paraffin-embedded tissue sections. Figure 3b shows the caspase-3 transcript expression in representative breast tissue samples from a normal and a cancer patient. The *in situ* RT–PCR analysis further confirmed the deficient status of caspase-3 transcript expression in breast tumor. The caspase-3 expression in normal breast sample was restricted mainly to the ductal epithelial and myoepithelial cells (Figure 3b).

Next, we determined the caspase-3 protein levels in breast cancer samples by using immunoblot analysis. No immunoreactive band corresponding to the authentic procaspase-3 protein (32 kDa) was evident in any of the six breast tissue samples (Figure 4A) that were negative for caspase-3 expression according to RT–PCR analysis (Table 1). Under similar conditions, however, the cell lysates from a prostate cancer LNCaP cell line that we used as a positive control, showed a strong immunoreactive band corresponding with procaspase-3 protein (Figure 4a). Similarly, the extracts from breast tissue samples that exhibited caspase-3
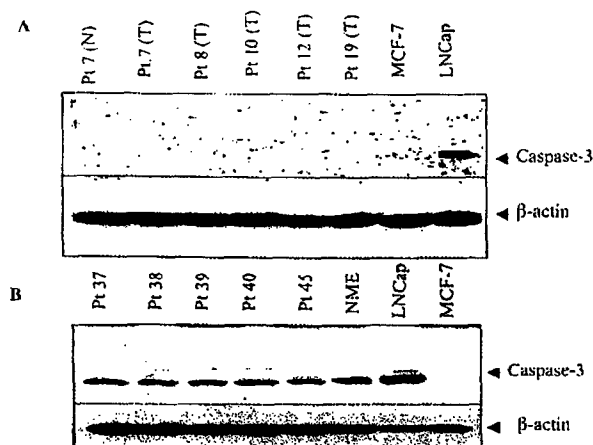
**Figure 4** Western blot analysis of breast tissue samples for cas-pase-3 protein expression. Breast carcinoma (T) or adjacent nor-mal (N) tissue samples obtained from breast cancer patients were homogenized in buffer, and 60 $\mu$g of homogenate protein was separated on an sodium dodecyl sulphate polyacrylamide gel electrophoresis. The membranes were probed with an anti-cas-pase-3 antibody. (a) Breast tissue samples that showed no detect-able caspases-3 transcript levels by RT–PCR analysis (Table 1). (b) Breast tissue samples that showed detectable caspase-3 tran-script levels. The finite-life-span NME and LNCap cells showed a strong 32 kDa immunoreactive band corresponding with cas-pase-3 protein. Wild-type MCF-7 cells completely lacked cas-pase-3 protein expression. The membranes were stripped and reprobed with anti-$\beta$ actin antibody to confirm equal protein loading and even transfer. Pt denotes patient, (N) denotes adja-cent normal tissue sample, ('T') denotes tumor sample, and NME denotes normal mammary epithelial cells

transcript expression by RT–PCR (Table 1), also showed a 32 kDa immunoreactive band, corresponding to procaspase-3 protein (Figure 4b). A similar 32 kDa band was detected in extracts from the NME cells but MCF-7 cells completely lacked its expression, probably due to the 125 bp deletion in the transcript.

*Effect of caspase-3 reconstitution on apoptosis*

In order to determine the significance caspase-3 downregulation in breast cancer, we compared the ability of caspase-3-deficient and caspase-3 reconsti-tuted MCF-7 cells to undergo apoptosis in response to calcium ionophore A23187 and staurosporine treat-ment. To reconstitute caspase-3, MCF-7 cells were transfected with pBabe/puro retroviral vector plasmid encoding a full-length procaspase 3 cDNA (pBS-CPP32) or empty vector (pBS) (Figure 5a). Flow cytometry analysis of ionophore A23187 (2 $\mu$M, 48 h) and staurosporine (50 nM, 48 h)-treated MCF-7 cells revealed significant apoptosis (accumulation in sub-G1 phase) in pBS-CPP32-transfected MCF-7 cells; MCF-7/pBS control cells under similar conditions showed no appreciable apoptosis (Figure 5b,c). Similarly, the multidrug resistant MCF-7/DOX cells which contain constitutive expression of caspase-3 protein (Devarajan et al., 2002), showed comparable apoptosis to pBS-CPP32 transfected MCF-7 cells in response to A23187

(Figure 5b) or staurosporine treatment (Figure 5c). Moreover, the treatment of MCF-7/DOX and MCF-7/pBS-CPP32 cells with ionophore A23187 yielded active fragments of caspase-3, as determined by a decrease or disappearance of the procaspase-3 32 kDa band by Western blot (data not shown). As previously reported (Yang et al., 2001), the MCF7/pBS-CPP32 cells exhibited at least 2–3-fold' higher sensitivity towards doxorubicin-induced killing effect when compared with the control MCF-7/pBS cells (data not shown). These results suggested that lack of caspase-3 could render breast cancer cells resistant to doxorubicin as well as to other apoptotic stimuli.

Finally, to determine whether the loss of caspase-3 expression represented a selective lesion in breast cancer patients, we tested some caspase-3-negative tumor samples for the expression of other caspases. The results shown in Figure 6 demonstrated that, despite a complete lack of procaspase-3 expression, the breast tumors had high levels of procaspase-9 and -8 expression. These results suggested that the loss of caspase-3 expression represents a selective event in breast cancer cells.

**Discussion**

It is now becoming apparent that many drugs can kill tumor cells by activating common apoptotic pathways (Hickman, 1996; Clynes et al., 1998). Thus, somatic, genetic or epigenetic alterations that disable apoptosis can produce multidrug resistance. In this study, we demonstrate one such defect in breast cancer cells that can attenuate drug-induced apoptosis. A significant majority of the human breast tumors tested lacked caspase-3 expression. Caspase-3 is a member of the cysteine protease family, which plays a central role in the execution of apoptosis. We studied caspase-3 expression in breast cancer cells partly because of its important role in apoptosis but more so because in several of the breast cancer cell lines that we studied it was either altered or lost (Figure 1). Additionally, caspase-3 activation has been detected in response to a variety of apoptotic stimuli, including chemotherapeu-tic agents, irradiation, and cytokines (Salvesen and Dixit, 1997). Conversely, selective inhibition of caspase-3 has been associated with inhibition of cell death (Hasegawa et al., 1996; Silke et al., 2001). These observations imply that loss of caspase-3 expression may render breast cancer cells resistant to chemother-apy and radiation therapy. Indeed, MCF-7 cells, which lack caspase-3 expression as a result of a functional deletion mutation in the *CASP3* gene, are relatively insensitive to cisplatin, doxorubicin and etoposide. However, reconstitution with caspase-3 rendered MCF-7 cells sensitive to these and various other apoptotic stimuli (Figure 6) (Yang et al., 2001). Conversely, despite the lack of caspase-3 expression, MCF-7 cells undergo morphological apoptosis in response to a variety of agents (Eck-Enriquez et al., 2000). It is likely that these agents can induce apoptosis
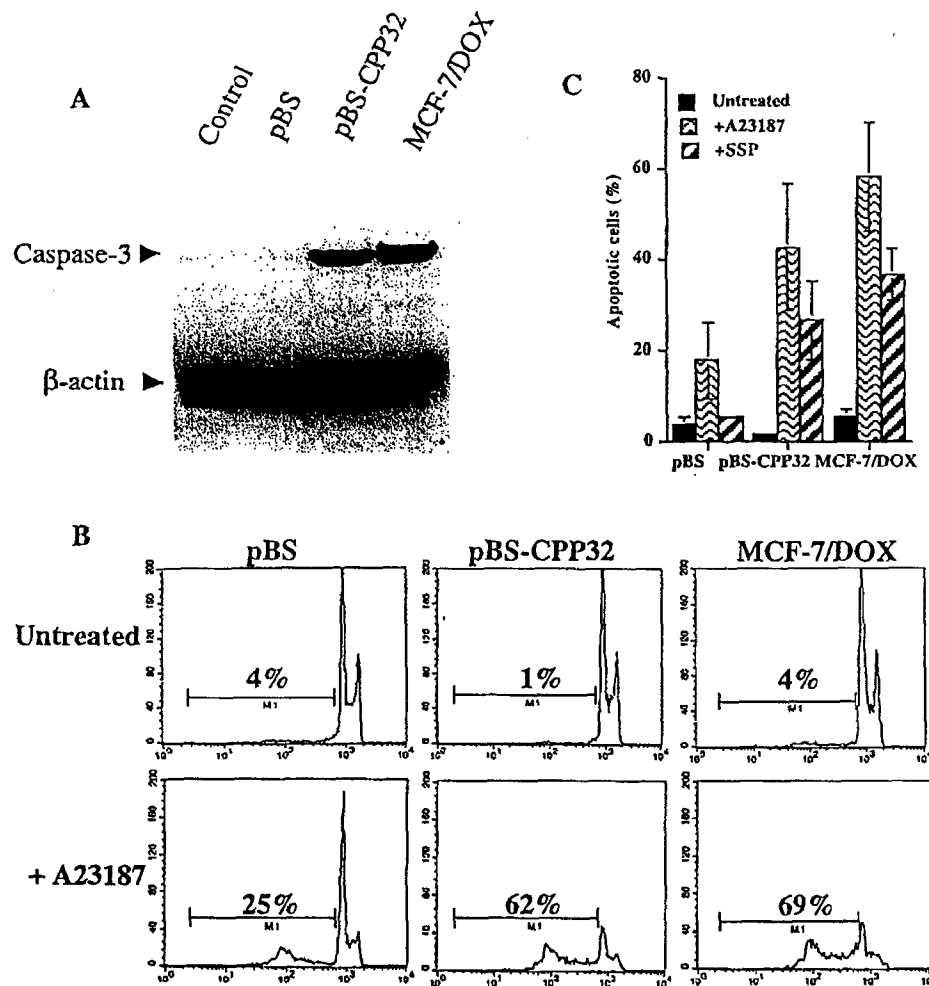
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**Figure 5** The effect of caspase-3 reconstitution on ionophore A23187 and SSP-induced apoptosis in MCF-7 cells. (a) Protein levels of caspase-3 in parental MCF-7 cells and vector-alone (pBS) or caspase-3 (p-CPP32)-transfected MCF-7 cells were detected by Western blot. Drug-resistant MCF-7 (DOX) cells served as a positive control for caspase-3 expression. (b) Untreated and ionophore A23187 (2 μM)-treated MCF-7 cells were subjected to flow cytometry analysis after staining with acridine orange. Plot showing DNA content of cells incubated for 48 h in medium alone (top panel) or medium containing ionophore (bottom panel). Cells that exhibit the sub-G1 DNA levels are considered apoptotic. (c) Following the incubation in medium alone (untreated) or medium containing ionophore A23187 (2 μM) or SSP (50 nM), cells were subjected to flow cytometry analysis. The mean ± s.d. of percent apoptotic cells that exhibited sub-G1 DNA levels after 48 h of treatment are shown

either in a caspase-independent manner (for example via AIF; Cande et al., 2002) or may involve alternative downstream caspases, such as caspase-6 and -7 (Liang et al., 2001). Nevertheless, drug-resistant breast cancer cells could be rendered sensitive to epirubin, taxol, and ectoposide simply by overexpressing the caspase-3 protein (Friedrich et al., 2001).

Another interesting aspect of this study was the observation that caspase-3 expression is also lost in normal looking breast parenchyma from breast cancer patients. Out of the twelve patients from whom such samples were obtained in this study, only one (patient #10) showed detectable levels of caspase-3 expression by RT–PCR analysis (Table 1). In contrast, seven (patients #34–40) out of ten normal breast parenchyma tissue samples studied from patients who had

undergone surgical tumor resection, showed normal levels of caspases-3 expression. The two normal breast tissue samples obtained during breast reduction surgery (patients #45 and 46) showed normal caspase-3 expression levels by RT–PCR (Figure 2c) and immunoblotting (Figure 4b). Interestingly, the normal contralateral breast tissue sample from one patient (patient #4) exhibited a detectable level of caspase-3 transcript (Table 1); whereas both the tumor sample and the adjacent normal parenchyma sample from the same patient but obtained from the tumor-affected side showed a complete loss of caspases-3 expression. Moreover, substantial caspase-3 expression levels were observed in breast tissue samples obtained from three individuals (patients #42–44) who did not have neoplastic breast disease.
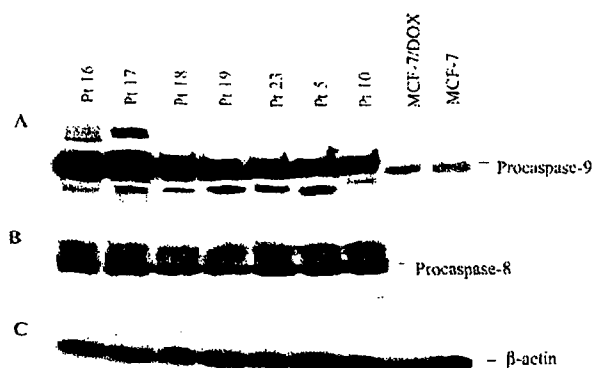
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Figure 6 Western blot analysis showing caspase-8 and -9 protein expression in caspase-3-negative breast tumor samples. Sixty micrograms of homogenate protein was separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Each membrane was probed with specific antibodies against caspase-9 (a) or caspase-8 (b). One of the membranes (a) was stripped and reprobed with anti-$\beta$ actin antibody to confirm equal protein loading and even transfer (c). Pt denotes patient

These results suggest that a lack of caspase-3 can attenuate apoptosis in response to certain stimuli, including chemotherapeutic drugs. Disruption of the intrinsic apoptotic pathways is frequently observed in cancer cells. For example, the tumor suppressor gene *p53* is inactivated in more than 50% of human cancers, resulting in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade (Harris, 1996). Conversely, the proteins that are known to serve as antiapoptotic survival signals, such as *bcl-2, bcl-x, ras,* and *c-abl,* are often overexpressed in tumor cells and cell lines (Martin and Green, 1995). The correction of these alterations is associated with an increased propensity for the tumor cells to undergo apoptosis and re-establishes chemosensitivity (Johnstone *et al.,* 2002; Wallace-Brodeur and Lowe, 1999).

Importantly, the ability of cells to evade apoptosis is one of the essential hallmarks of cancer cells, the skill that can help them breach the anticancer defense mechanisms (Hanahan and Weinberg, 2000). Thus, loss of expression of caspase-3 and/or other caspases may serve as a pivotal step in the survival of mutated somatic cells. Unlike that of caspase-3, the expression of caspase-8 and -9 was normal in breast tumor samples in the present study (Figure 6). However, complete inactivation of the gene for caspase-8 was recently reported in neuroblastomas, a childhood tumor of the peripheral nervous system (Teitz *et al.,* 2000). Additionally, caspase-8-null neuroblastoma cells were resistant to doxorubicin- and death receptor-induced apoptosis. Furthermore, more than 80% of prostate tumors exhibit a complete lack of caspase-1 protein expression and reduced levels of caspase-3 expression (Winter *et al.,* 2001). Similarly, *Apaf-1,* a cell-death effector protein that acts in association with cytochrome-*c* and caspase-9, its expression is lost in the majority of metastatic melanomas (Soengas *et al.,*

2001). These observations suggest that attenuation of apoptosis due to the inactivation or silencing of caspases may represent an important mechanism of cell survival and chemoresistance. .

Our present findings are in contrast with those of an earlier report by Nakopoulou *et al.* (2001). These authors employed an immunohistochemical assay to study caspase-3 protein expression in breast tumor samples and concluded that 75% of the breast tumors had higher levels of expression of this protein than the non-neoplastic breast tissue. Contrary to these findings, our results suggested that downregulation and deficiency of caspase-3 expression occurs in breast cancer cells and may represent a potential mechanism of cell survival. The observation of high caspase-3 expression by Nakopoulou *et al.* (2001) using immunohistochemical assay can be explained by possible cross reactivity of anti-caspase-3 antibody with some non caspase-3 protein in the breast tissue sections.

Finally, based on our preliminary results using the total RNA isolated from the breast, ovarian, cervical, and uterine carcinomas, it is likely that a similar loss or downregulation of caspase-3 expression may occur in other gynecologic tumors (Figure 3a). At this point, we do not know the mechanisms that may underlie the loss of caspase-3 expression. CpG island hypermethylation, which is known to result in the silencing of several key genes in breast cancer (Huang *et al.,* 1999), might play a role. Indeed, silencing of the caspase-8 and caspase-1 genes was recently shown to result from hypermethylation in childhood neuroblastoma and renal cancer cells, respectively (Teitz *et al.,* 2000; Ueki *et al.,* 2001). It is equally possible that some functional deletions in the *CASP3* gene may result in the loss of its expression. For instance, a 124 bp deletion in the caspase-3 transcript in MCF-7 cells (Janicke *et al.,* 1998) as well as in ZR-75T and BT-20T breast cancer cell lines (unpublished observations) results in a complete loss of caspase-3 protein expression (Figure 1). Moreover, we recently observed that some breast cancer patients harbor a 121 bp deletion (bp$_{483\rightarrow604}$) in the active-site region of the caspase-3 transcript (unpublished observations). A similar deletion in the C-terminal end of the caspase-3 (casp3$\Delta$C) transcript was observed by Huang *et al.* (2001) in a human colon carcinoma cell line; this deletion results from alternative mRNA splicing, and its product acts as a dominant, negative regulator, rendering the cells resistant to apoptosis.

In conclusion, our results suggest that the tumor cells as well as the normal parenchyma surrounding the tumor lack caspase 3 expression in the majority of breast cancer patients. As one would expect, loss of expression or function of this key caspase, can render breast cancer cells resistant to apoptosis in response to certain apoptotic stimuli including chemotherapeutic drugs and thus may affect the outcome and prognosis of the disease. These findings may have important clinical implications in terms of using caspase-3 not only as a marker of disease but also as a therapeutic target for breast cancer.

## Materials and methods

### Cell lines and tissue samples

The human breast cancer cell lines T47D, MDA-MB-361, MDA-MB-468, BT-474, SKBR-3, BT-20, MDA-MB-157, and MDA-MB-436 and the mammary epithelial cell line, MCF10A were obtained from and maintained according to the instructions provided by the American Type Culture Collection (Rockville, MD, USA). In addition, the MDA-MB-134, Hs854T, HCC1954, HCC1428, DU4475, BT-549, and BT-483 breast cancer cell lines were provided by Dr J Dai (Yuan *et al.*, 2001), and BT-20T and ZR-75T cancer cell lines were provided by Dr K Keyomarsi (Chen *et al.*, 2000). Two normal mammary epithelial (NME) cell lines having a finite life span were purchased from Clonetics (San Diego, CA, USA). The drug-resistant MCF-7/DOX cell line was established via continuous culture of MCF-7 cells in the presence of doxorubicin as described previously (Devarajan *et al.*, 2002). The caspase-3 deficient MCF-7 cells were infected with either pBabe/puromycin retroviral vector alone (pBS) or vector containing full-length caspase-3 cDNA insert (pBS-CPP32), as described previously (Yang *et al.*, 2001).

Primary breast tumor and normal breast parenchyma samples (4–6 mm$^3$ thick) were obtained from patients who had undergone breast surgery at The University of Texas MD Anderson Cancer Center. A total of 46 patients, including 31 having breast adenocarcinoma (four of whom had received preoperative chemotherapy), 10 with a history of breast cancer, three having benign breast disease, and two undergoing breast reduction surgery were enrolled in this pilot study. The median age of the patients was 55 years. The samples were obtained with the patients' consent following approval by the Institutional Committee for the Welfare of Human Subjects. Microscopic examination of representative sections of each sample after staining with hematoxylin and eosin stain confirmed the presence of carcinoma cells in greater than 80% of the sample area. The samples were either directly collected in RNAzol for immediate isolation of total RNA or snap frozen and stored at −80°C for later use.

### Flow cytometric analysis of apoptotic cells

To determine the apoptosis, $1-2 \times 10^6$ cells were seeded into T-75 tissue culture flasks the day before treatment with 2 µM ionophore A23187 or 50 nM staurosporine (SSP) (both from Sigma). After 48 h treatment, free-floating cells were removed from the flasks and pooled with adherent cells obtained by trypsinization from the respective flasks. Pooled cells were permeabilized and incubated with 8 µg/ml acridine orange (Polysciences, Warrington, PA, USA). Cell fluorescence was measured using the logarithmic scale of the FACScan flow cytometer with a 488 nm excitation of a 15 mW argon laser and filter settings for green (530 nm; DNA) and red (585 nm; RNA) fluorescence. Ten thousand events were stored and run for analysis following the elimination of cell debris and doublets. The percentage of cells in the 'sub G1' region defined the proportion of apoptotic cells in the tested populations.

### Reverse transcriptase-polymerase chain reaction (RT–PCR) analysis

RT–PCR analysis of caspase-3 transcript was carried out as described previously (Devarajan *et al.*, 2002). Briefly, total RNA was isolated from tissue samples or cells using the TRIzol method (Guo and Kyprianou, 1999). RT–PCR was

performed using 5 µg of total RNA and the Superscript II reverse transcript kit (Life Technologies, Grand Island, NY, USA) in an amplification cycler (Perkin-Elmer, Wellesley, MA, USA). The sequences of the caspase-3-specific primers used were as follows: sense, 5'-TTAATAAAGGTATC-CATGGAGAACACT-3'; and antisense, 5'-TTAGTGATA-AAAA TAGAGTTCTTTTGTGAG-3'. RT–PCR was performed at 94°C for 2 min, 33 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 5 min. The integrity of the RNA used for RT–PCR was confirmed using β-actin synthesis as a positive control reaction (Haidar *et al.*, 2000). The amplified RT–PCR products were separated on a 1% agarose gel, stained with ethidium bromide for visualization, and photographed under ultraviolet illumination. For complementary DNA (cDNA) sequencing, the PCR products were subcloned into pCR2.1 plasmid (Invitrogen, Carlsbad, CA, USA) and sequenced in both directions using an automated DNA sequencer (310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA).

### Northern blot analysis

Total RNA was isolated from primary breast tumor samples as described above. Also, to study caspases-3 expression in other gynecological tumors, total RNA isolated from human breast, cervical, ovarian, and uterine tumor tissues was purchased from Clontech Laboratories (Palo Alto, CA, USA). Ten micrograms of total RNA was mixed with the sample buffer (2.2 M formaldehyde, 50% formamide, 50 mM MOPS (pH 7.0) and 1 mM EDTA), denatured via heating at 65°C for 10 min, resolved on 1.5% agarose/formaldehyde gels, and transferred to a Hybond nylon membrane. In addition, nylon membrane containing messenger RNA (mRNA) isolated from various normal human tissues was purchased from Clontech Laboratories (Multiple Tissue Northern (MTN$^{TM}$) blot). Caspase-3-specific probe of 824 base pairs (bp) was generated using human mammary epithelial cDNA and caspase-3-specific primers. The PCR product of caspase-3 was oligolabeled with $^{32}$P-α-dCTP by random labeling (Boehringer Mannheim Corp., Indianapolis, IN, USA). Hybridizations were carried out overnight at 42°C using random primed labeled caspase-3 probe in Quikhyb (Stratgene, La Jolla, CA, USA) in accordance with the manufacturer's instructions. Furthermore, filters were exposed to autoradiographic film for up to 5 days. To test for the uniform loading of the samples, blots were stripped and reprobed using a cDNA probe for the human 18S rRNA or GAPDH.

The caspase 3 transcript in few selected samples was also determined by using an adaptation of RT–PCR *in situ* (RT–PCR IS) as described by Bagasara *et al.* (1993). The 5'-TTAATAAAGGTATCCATGGAGAACACT-3' primer was labeled with digoxigenin (Sigma Genosys, The Woodland, TX, USA) and PCR was performed for 35 cycles at 94°C for 50 s, at 55°C for 30 s, and at 72°C for 1 min.

### Western blot analysis

PBS-washed cells were lysed by sonication in ice-cold buffer A (20 mM Tris-buffered saline (pH 7.4) containing 1 mM EDTA, 14 mM 2-mercaptoethanol, and 1 µM phenylmethyl-sulfonyl fluoride). Lysed cells were centrifuged at 14 000 r.p.m. for 10 min to remove cell-debris. Protein extracts from breast cancer tissue samples were prepared by either directly grinding the tissue samples using a prechilled mortar and pestle in buffer A or extensively dialyzing the tissue extracts left behind after the total RNA extraction

against phosphate-buffered saline containing 0.1% sodium dodecyl sulfate (SDS) (Chomczynski, 1993). The dialyzed extracts were then concentrated using Centricon cartridge filter (10 kDa cut-off; Amicon Bioseparations, Bedford, MA, USA), and the protein content in cell and tissue extracts was determined using BioRad dye reagent I (Richmond, CA, USA). Equal amounts of protein (30–60 μg) were subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE), and the proteins were transferred onto nitrocellulose membranes. After overnight blocking with 5% nonfat dry milk, the membranes were probed with an anti-caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:2000, or anti-caspase-8 or anti-caspase-9 antibody (Cell Signaling Technology, Beverly, MA, USA) at a dilution of 1:2000. All of the membranes were stripped

and reprobed with an anti-$\beta$-actin antibody (Sigma Chemical Co, St. Louis, MO, USA) at a dilution of 1:2000. The Amersham Pharmacia Biotech ECL system (Piscutaway, NJ, USA) was used to detect the antigen-antibody reaction.

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